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Differentiation of rat adipose tissue-derived mesenchymal stem cells towards a nucleus pulposus-like phenotype *in vitro*

XIE Li-wei解礼伟, FANG Huang方煌, CHEN An-min陈安民 and LI Feng 李锋*

Objective: To differentiate rat adipose tissue-derived mesenchymal stem cells (ADSCs) into cells with a nucleus pulposus-like phenotype *in vitro*, so as to lay a foundation for the cell-based transplantation therapy of degenerated intervertebral discs.

Methods: Rat ADSCs were isolated only from the subcutaneous inguinal region and purified by limited dilution. ADSCs of the third passages were analyzed by fluorescence activated cell sorter (FACS) to detect the cell surface markers (Sca-1, CD44, CD45, CD11b). To induce ADSCs towards a nucleus pulposus-like phenotype, ADSCs were immobilized in 3-dimensional alginate hydrogels and cultured in an inducing medium containing transforming growth factor-beta1 (TGF-β1) under hypoxia (2% O_2), while control groups under normoxia (21% O_2) in alginate beads in medium with or without the presence of TGF-β1. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was carried out to evaluate phenotypic and biosynthetic activities in the process of differentiation. Meanwhile, Alcian blue staining were used to detect the formation of sulfated glycosaminoglycans (GAGs) in the differentiated cells.

Results: The purified ADSCs were fibroblast-like and proliferated rapidly *in vitro*. The flow cytometry showed that ADSCs were positive for Sca-1 and CD44, negative for CD45 and CD11b. The results of RT-PCR manifested that the gene expressions of Sox-9, aggrecan and collagen II, which were chondrocyte specific, were upregulated in medium containing TGF-β1 under hypoxia (2% O_2). Likewise, gene expression of HIF-1a, which was characteristics of intervertebral discs, was also upregulated. Simultaneously, Alcian blue staining exhibited the formation of many GAGs.

Conclusions: The approach in our experiment is a simple and effective way to acquire a large quantity of homogenous ADSCs. Rat ADSCs can be differentiated into nucleus pulposus-like cells. ADSCs may replace bone marrow mesenchymal stem cells as a new kind of seed cells in regeneration of degenerated intervertebral discs using cell transplantation therapy.

Key words: ***Mesenchymal stem cells; Transforming growth factor-beta1; Adipose tissue; Cell differentiation***

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Diseases related to intervertebral disc degeneration (IDD) are very common in clinic, and the morbidity is increasing as a consequence of population aging. Current treatments, both conservative and invasive, by either temporarily decreasing the axial load of intervertebral discs or by decompression via removing the degenerated intervertebral discs and stabilizing the spine, aim at symptomatic relief. However, these treatments can not restore the normal structure of degenerated intervertebral disc, because they have not focused on the key factor that degeneration of intervertebral disc is caused by decrease of nucleus pulposus cells and the subsequent decrease of proteoglycan in the extracellular matrix (ECM).[1] Fortunately, stem cell-mediated cell transplantation can reconstruct the normal structure and function of degenerated intervertebral disc by supplementing the loss of nucleus pulposus cells and promoting the formation of ECM.[2,3,4] Risbud et al[5] once found that rat bone marrow mesenchymal stem cells (BMSCs) can be differentiated towards nucleus pulposus (NP)-like cells. Nevertheless, compared with BMSCs, adipose tissue-derived mesenchymal stem cells (ADSCs) have an equal potential of differentiation into osteocytes, adipocytes,

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Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China (Xie LW, Fang H, Chen AM and Li F)

*Corresponding author: E-mail: fanghuangtjh@126.com

chondrocytes and so on, but they can be obtained in a less invasive way, which have attracted more and more attention from researchers.[6] Our aim is to differentiate rat ADSCs into cells with a nucleus pulposus-like phenotype on the basis of isolation, purification and identification of ADSCs, so as to provide the reference for the biotherapy of the IDD-related diseases.

METHODS

Isolation and culture of ADSCs

Adipose tissue was dissected from lymph nodes and capillary vessels in subcutaneous inguinal region of male Sprague-Dawley rats (200 g), and washed three times with phosphate-buffered saline (PBS), then finely minced into small pieces and immersed in PBS. The tissue debris was centrifuged at 2 000 r/min for 10 minutes. The supernatant was reserved, resuspended with PBS and centrifuged at 2 000 r/min for 10 minutes. Then the supernatant was digested at 37°C for 2 hours in 0.1% collagenase and prepared in Dulbecco modified Eagles medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). The tube was shaked every half an hour, and then centrifuged at 1 500 r/min for 5 minutes to sediment cells while the supernatant was discarded. The cell pellet was then suspended with PBS, centrifuged at 1 500 r/min for 5 minutes and resuspended in DMEM/F12 with 10% FBS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) before being plated into a 96-well plate. Twenty-four hours later, nonadherent cells were removed by PBS washing and subsequent medium changes. The adherent cells formed colonies that were expanded in monolayer and the cells would be released by 0.25% trypsin and replated when reaching confluence. The cultured cells were maintained with 5% CO_2 at 37°C and the medium was changed every 3 days. The third passages were preserved for experiment.

Identification of ADSCs

Fluorescence activated cell sorter (FACS) was used to analyze the surface markers of rat ADSCs. The third passages were trypsinized and resuspended in DMEM/F12 containing 10% FBS. Samples were counted, centrifuged and resuspended in PBS. The cells were placed into Eppendorf tube for 1×10^6 cells per 1.5 ml, washed twice with PBS and incubated for 1 hour at room temperature with the following FITC-conjugated antibodies (Peprortech Inc, USA): anti-rat Sca-1 (1:200), anti-rat CD44 (1:200), anti-rat CD45 (1:200) and anti-rat CD11b (1:200). In control groups, the cells were incubated in PBS without antibodies. The samples were then washed twice with PBS and analyzed by FASC.

Differentiation of ADSCs towards a NP-like phenotype

ADSCs of the third passage were rinsed for three times with PBS and harvested with 0.25% trypsin. The obtained cells were counted and resuspended with 1.2% alginate solution, prepared with low-viscosity sodium alginate (Sigma, USA) in 0.9% NaCl and filtered through 0.22 μm filters, at a density of 1×10^6 cells/ml. The alginate-cell suspension was then expelled through a 22-gauge needle into the solution of 3.5% $CaCl_2$ prepared with 0.9% NaCl to cause the instantaneous formation of alginate hydrogels (2 mm in diameter). The needles were about 2 cm above the surface of the $CaCl_2$ solution and the gel time was 10 minutes. Subsequently, the alginate beads were washed slightly and thoroughly with PBS. To induce the differentiation of ADSCs in the microbeads towards a NP-like phenotype, the beads were cultured in a differentiating medium, containing 10% FBS in DMEM/HG supplemented with 10 ng/ml TGF-β1(R&D, USA), 100 nmol/L dexamethasone, 50 μg/ml L-ascorbic acid 2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline and 100 × ITS-liquid media supplement (Sigma, USA). The alginate beads were cultured at 37°C in a triphasic mini-incubator with 2% O_2 and 5% carbon dioxide. Control groups were ADSCs in alginate beads in differentiating medium at 21% O_2 and ADSCs in alginate beads in normal medium consisting of 10% FBS in DMEM/HG at 21% O_2. After 7 days of culture, cells in the microbeads were released by 55 mmol/L sodium citrate and prepared in 0.9% NaCl. The retrieved cells were then washed with PBS and used for RNA isolation.[7] Groups were as follows: Group A, ADSCs in alginate beads in normal medium consisting of 10% FBS in DMEM/HG at 21% O_2; Group B, ADSCs in alginate beads in differentiating medium at 21% O_2; and Group C, ADSCs in alginate beads in differentiating medium at 2% O_2.

Visualization of functional proteoglycan matrix

At the end of the 7-day induction, several microbeads from each group were taken for Alcian blue staining to visualize the formation of sulfated glycosaminoglycans (GAGs) in differentiated ADSCs, using an inverted

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microscope and a digital camera. The microbeads were washed three times firstly and then fixed with a Kalhes's fixator (6 ml formalin, 15 ml 95% ethyl alcohol, 1ml glacial acetic acid, 80 ml distilled water) for 20 minutes at room temperature, then washed with 50% ethanol and 70% ethanol, respectively. Subsequently, the microbeads were stained with 1% Alcian blue solution (1g alcian blue 8 GX, 100 ml 3% acetic acid solution) (Sigma, USA) for 30 minutes and rinsed in distilled water before observation.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the retrieved cells using TRNzol-A$^+$ (Tiangen, Beijing, China) according to the manufacturer's instructions. Then 1-2 micrograms of total RNA was reversely transcribed into cDNA and the reverse transcription (RT) system was designed as follows: 2 μl Oligo (dT) primers, 2 μg total RNA, add DEPC treated water to 12 μl; at 70°C for 5 minutes; 4 μl 5×RT buffer, 2 μl dNTP (10 mmol/L), 1μl RNaes ihibitor, 1μl RT Ace (Toyobo, Japan); at 42°C for 1 hour; at 95°C for 5 minutes. For polymerase chain reaction (PCR), the system was designed as follows: 5 μl 10×Taq buffer, 0.5 μl dNTP (10 mmol/L), 0.5 μl sense primer (20 μmol/L), 0.5 μl anti-sense primer (20 μmol/L), 2 μl cDNA, 0.5 μl Taq polymerase (Toyobo, Japan), add ddH_2O to 25 μl; at 4°C for 5 minutes; at 94°C for 45 seconds; at 55°C for 45 seconds; at 72°C for 1 minutes; 30 cycles; at 72°C for 10 minutes. Primers (Invitrogen Biotechnology Co, Ltd, Shanghai, China, Table 1) based on rat sequences were custom designed and synthesized by integrated DNA technologies. After electrophoresis in 1.5% agarose gel with ethidium bromide alongside a 100 bp DNA ladder, the PCR product was visualized by a Bio-Imaging System (Bio-Rad Co, USA). GAPDH was used to normalize the relative expression of the target genes.

Table 1. Primers for PCR

Genes	Primers	Primer sequence	Size (bp)
GAPDH	Forward	5'-AGAACATCATCCCTGCATCC-3'	398
	Reverse	5'-TTACTCCTTGGAGGCCATGT-3'	
Collagen II	Forward	5'-CACTCATCTGTTGTGATGAGTTCTCC-3'	175
	Reverse	5'-CAACACACACCAGCGCAGTTT	
Aggrecan	Forward	5'-GGGTGAGGTCTTTTATGCCA-3'	276
	Reverse	5'-GCTTTGCAGTGAGGATCACA-3'	
Sox-9	Forward	5'-TTGCTCGGAACTGTCTGGAA-3'	389
	Reverse	5'-CCTGCTCGTCGGTCATCTT-3'	
HIF-1a	Forward	5'-ACTATGTCGCTTTCTTGG-3'	195
	Reverse	5'-GTTTCTGCTGCCTTGTA-3'	

Statistical analysis

Data was represented as Mean ± SD and statistical analysis was carried out using analysis of variance (ANOVA) by post hoc LSD test. $P<0.05$ was considered statistically significant.

RESULTS

Culture of isolated ADSCs in monolayer

ADSCs cultured in monolayer had a fibroblast-like shape, with nucleus and nucleolus clearly visible under inverted phase contrast microscope. ADSCs expanded quickly in clones of whirlpool-like distribution could reach confluence in about two weeks for the primary passage and 7 days for subcultures. Notedly, cells acquired in this way had a high purity (Fig.1).

Results of identification of ADSCs

Flow cytometry analysis showed that rat ADSCs expressed a number of specific markers of stem cells, such as Sca-1 and CD44, while CD45 and CD11b, lymphohematopoietic markers, were expressed at very low levels. Therefore, we concluded that rat ADSCs were positive for Sca-1 and CD44 and negative for CD45 and CD11b (Fig.2).

Alginate beads and ADSCs in alginate beads

ADSCs in alginate hydrogels maintained a round morphology and showed no expansion during culture at the seeding density (Fig.3).

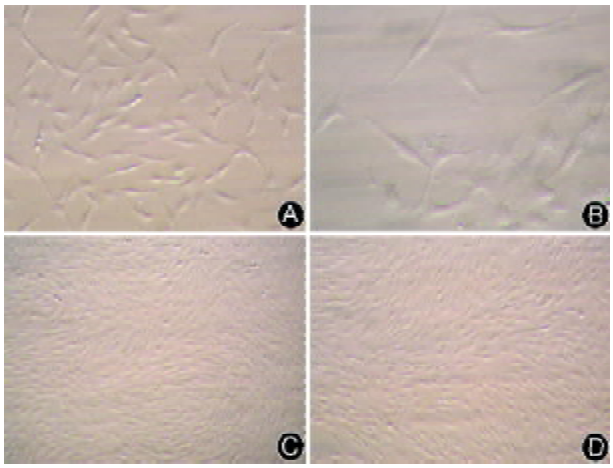

Fig.1. Morphology of ADSCs cultured in monolayer. **A:** primary passage 10 days after seeding, ×200; **B:** local magnification of primary passage, ×300; **C:** primary passage reaching confluence, ×100; **D:** third passage reaching confluence, ×100.

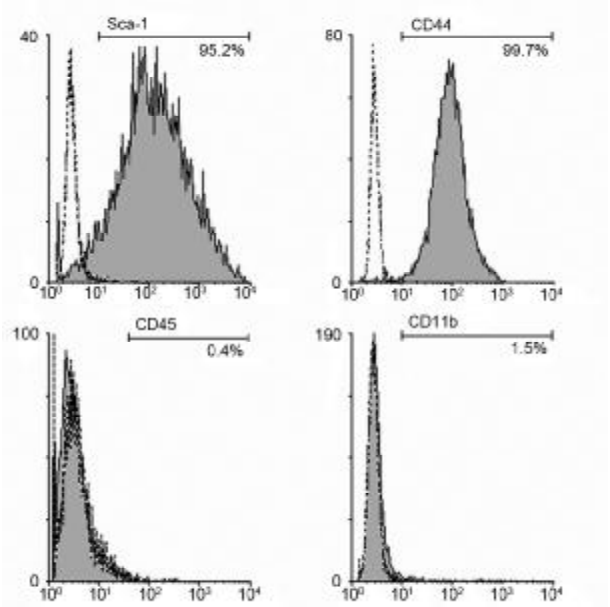

Fig.2. Flow cytometry analysis of rat ADSCs of third passage. Sca-1 and CD44 were expressed at very high level, while CD45 and CD11b at very low level.

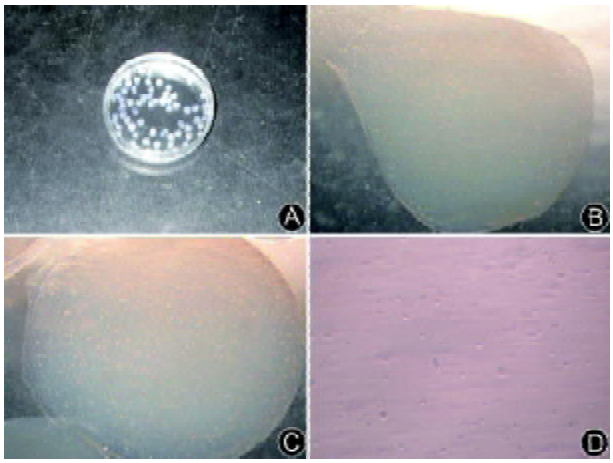

Fig.3. Alginate beads and rat ADSCs in alginate beads. **A:** alginate beads observed with naked eyes. **B, C:** ADSCs in the alginate beads, ×40. **D:** ADSCs in alginate beads, ×100.

Results of Alcian blue staining

GAGs, main components of extracellular matrix, secreted by ADSCs, were visualized by Alcian blue staining. Alginate beads in differentiating medium showed a higher formation of GAGs than that in normal medium. And the stronger intensity could be seen in alginate beads under hypoxia condition (Fig.4).

Results of RT-PCR

The results of RT-PCR indicated that ADSCs in alginate beads treated with differentiating medium showed a significant increase in the gene expression of collagen II, Sox-9 and aggrecan (P<0.05) and the expression in hypoxia groups was further elevated as compared with normoxia groups (P<0.05). Likewise, HIF-1a gene's expression was also upregulated in hypoxia groups (P<0.05) while there was no significant difference between groups under normoxia conditions (P>0.05, Fig.5).

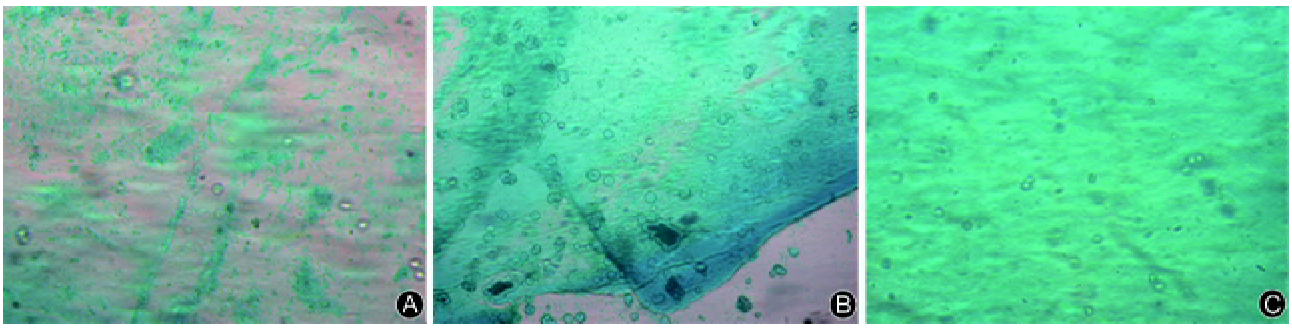

Fig.4. Alcian blue staining. **A:** ADSCs in alginate beads in normal medium at 21% O_2. **B:** ADSCs in alginate beads in differentiating medium at 21% O_2. **C:** ADSCs in alginate beads in differentiating medium at 2% O_2. Alginate beads in differentiating medium showed a higher formation of GAGs than alginate beads in normal medium. And stronger intensity could be seen in alginate beads in hypoxia conditions.

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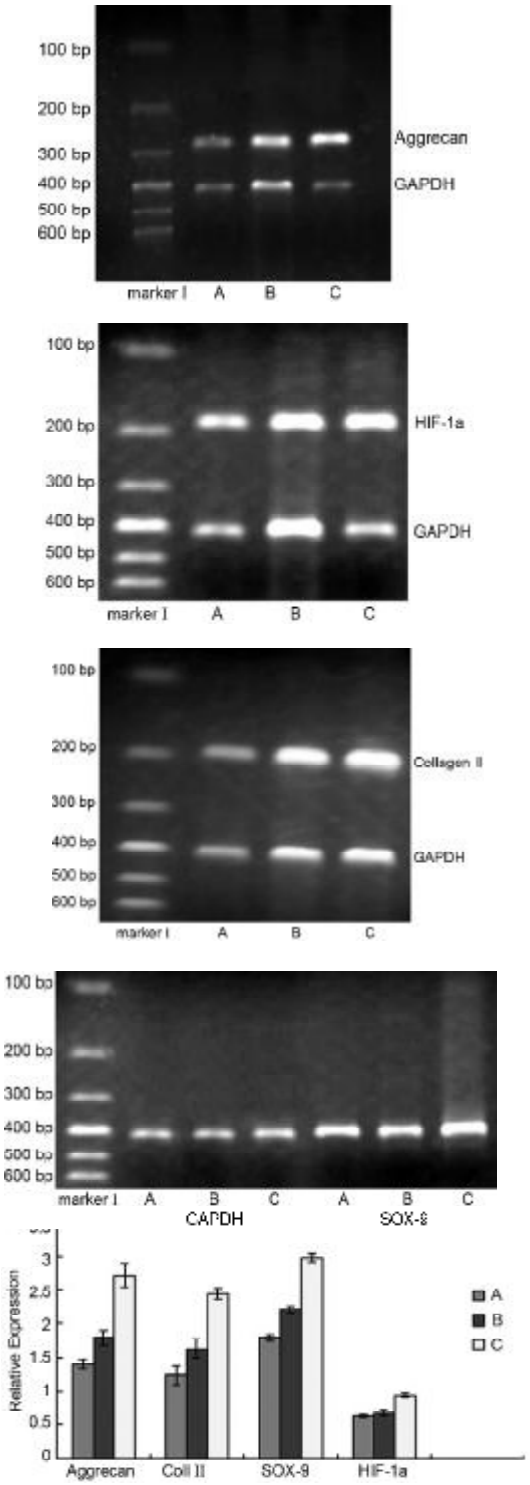

Fig.5. The results of RT-PCR. Group A: ADSCs in alginate beads in normal medium at 21% O_2; Group B: ADSCs in alginate beads in differentiating medium at 21% O_2; Group C: ADSCs in alginate beads in differentiating medium at 2% O_2.

DISCUSSION

Tissue engineering of nucleus pulposus has always been the focus of stem cell regeneration of degenerated intervertebral discs.[2-4] Risbud et al[5] once proved that under hypoxia conditions (2% O_2), in a chondrogenic medium within alginate beads, rat BMSCs can be differentiated towards a NP-like phenotype. However, the way to obtain BMSCs is usually invasive, while ADSCs can be easily harvested from patients by a simple and minimal invasive approach. The two kinds of stem cells have an equal potential to be differentiated into osteocytes, adipocytes, chondrocytes and so on.[8-10] Therefore, we intended to differentiate rat ADSCs into nucleus pulposus.

The results of RT-PCR showed that ADSCs in alginate beads treated with differentiating medium had a significant increase in the gene expression of collagen II, Sox-9 and aggrecan, and the expression was further elevated in hypoxia groups as compared with normoxia groups. Correspondingly, the alcian blue staining was also more intense in hypoxia groups. Likewise, the HIF-1a gene's expression was also upregulated in hypoxia groups while there was no significant difference between groups under normoxia conditions. Previous studies showed that genes like collagen II, Sox-9 and aggrecan, were chondrocyte-specific.[11] So it was concluded that rat ADSCs had been differentiated into chondrocyte-like cells in the defined differentiating medium. Studies also revealed that nucleus pulposus could be regarded as chondrocyte-like cells because of the expression of chondrocyte-specific genes,[11] such as collagen II, Sox-9 and aggrecan. Meanwhile, gene HIF-1a was a phenotypic characteristic of the nucleus pulposus,[12, 13] indicating that ADSCs had been differentiated towards a NP-like phenotype. GAGs, main components of extracellular matrix, formed by the differentiated ADSCs in inducing medium, were visualized by Alcian blue staining, which demonstrated the higher formation of GAGs in hypoxia than that in normoxia. Therefore, we concluded that rat ADSCs could be differentiated into functional NP-like cells.

In our study, three-dimensional alginate beads were used to imitate the micro-environment of the nucleus pulposus cells, which also facilitated the detection of GAGs in the extracellular matrix. And because intervertebral discs were avascular tissues with a low local oxygen tension,[14] 2% O_2 was applied in our study. The inducing medium containing TGF-β1 was used to make sure that ADSCs differentiated along a chondrogenic direction.[15]

In addition, the results of RT-PCR demonstrated that hypoxia could promote chondrogenic potential of ADSCs, which could be inferred from the outcome that the expression of chondrocyte-specific genes was upregulated in hypoxia groups compared to the groups in differentiating medium under normoxia conditions. And it was consistent with previous studies.[16] Besides, rat ADSCs in our experiment were positive for mesenchymal stem cell markers Sca-1 and CD44, negative for CD45 and CD11b,[17,18] which was proved by flow cytometry analysis in our study. This method was effective to acquire ADSCs of high purity.

However, the differentiation mechanism is not clear and whether there is the same change at the protein level still needs further research. According to Risbud et al[5], MAPK signaling pathway plays an important role in the differentiation of BMSCs towards NP-like cells. But it is not sure whether there is a similar change in the differentiation of ADSCs. If the same results can be detected at the protein and molecular level, there exists the probability that ADSCs will replace BMSCs in the stem cell regeneration of degenerated intervertebral discs.

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